IMPACT OF STRAIGHT CHAIN FATTY ACIDS ON STAPHYLOCOCCUS AUREUS GROWTH AND BIOFILM PRODUCTION

by

KENDALL CLERICI

(Under the Direction of Kelly Marie Hines)

ABSTRACT

Over the past century, the rapidly rising rates of antibiotics resistance has led a global health crisis being linked with an estimated over 2.5 million infections and 35,000 deaths in the United States from bacteria such as methicillin resistant *Staphylococcus aureus* (MRSA). MRSA infections have been linked lipid rich environments such as skin and heart presenting unexplored impacts on biofilm formation. This study investigates the role of palmitic (16:0) and oleic acid (18:1), two straight chain biologically prominent fatty acids (FA), on the biofilm formation of JE2, parent strain of *S. aureus,* and *fakA::Tn* linked with reduced exogenous FA incorporation. Additionally, growth curves were performed with oleic acid and three derivative whiles being challenged with daptomycin, an antibiotic for MRSA. The results point towards a FA dependent and mutant effect on for both biofilm formation and growth curves signifying a complex interplay is occurring between these systems.

INDEX WORDS: Straight chain fatty acids, *S. aureus*, *S. aureus* biofilm associated infections

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DEDICATION

I dedicate my thesis to my family, friends, and everyone who believed in me. I am so grateful for your constant love and support. This wouldn't have been possible without you. Thank you for standing by my side as I finish this journey.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

ABBREVIATIONS

TSB: Tryptic soy broth

$O.D._{600}$: Optical density at 600 nanometers

$O.D._{655}$: Optical density at 655 nanometers

$O.D._{530}$: Optical density at 530 nanometers

PBS: Phosphate buffer solution

EtOH: Ethanol

IPA: Isopropanol

PA: Palmitic acid

OA: Oleic acid

SA: Stearic acid

LA: Linoleic acid

DMSO: Dimethyl sulfoxide

70/10/20 EtOH/IPA/H_2O: 70% ethanol, 10 % isopropanol, 20% water

10-HSA: 10-hydroxystearic acid

cis-VA: *cis*-Vaccenic acid

cis-9,10-MOA*: cis*-9,10-methyleneoctadecanoic acid

fak: Fatty acid kinase complex

fakA: Fatty acid kinase A

fakB: Fatty acid kinase B

fakB1: Fatty acid kinase B1

fakB2: Fatty acid kinase B2

S. aureus: Staphylococcus aureus

EPS: Extracellular polymetric substances

PBP-2a: penicillin-binding protein 2a

Dap15: 15 ug/mL daptomycin

Dap30: 30 ug/mL daptomycin

G3P: Glycerol-3-phosphate

NAM: N-acetylmuramic

NAG: N-acetylglucosamine

PG: Phosphatidylglycerol

CL: Cardiolipin

L-PG: Lysly-phosphatidylglycerols

DGDG: Diglycosyldiacylglycerols

QRDR: Quinolone resistant-determining region

Enoyl-ACP: Enoyl-acyl carrier protein

Acyl-ACP: Acyl carrier protein

PA: Phosphatidic acid

Acyl-PO_4: Acyl phosphate

CTP: Cytidine triphosphate

CHAPTER 1

CHARACTERISTICS OF STAPHYLOCOCCUS AUREUS

1.1 Staphylococcus aureus (S. aureus)

S. aureus is a gram-positive bacterium that has a thick peptidoglycan layer and lacks an outer membrane.[1] The cells of *S. aureus* are sphere-shaped (cocci), the cells are yellow, and they often cluster together.[2] *S. aureus* has a cell membrane with a phospholipid bilayer, and a thick cell wall comprised of peptidoglycan that maintains the structure of the cell. *S. aureus* often lives on the skin of healthy individuals, but it can cause numerous infections. *S. aureus* can cause skin infections, pulmonary infections, and urinary tract infections. *S. aureus* can form biofilm, which often leads to the bacteria entering the bloodstream, which can cause sepsis.[2]

1.2 Antibiotic Resistant Bacteria

The ESKAPE pathogens are a group of bacteria that are increasingly becoming more resistant to antibiotics.[3] *S. aureus* is an ESKAPE pathogen, and these infections are difficult to contain because they spread through skin-to-skin contact, surfaces that are contaminated, and even water.[4] The rapid spread of these infections is greatly impacting global health and the global economy, with some countries spending millions of dollars attempting to fight these infections.[5]

Antibiotics have become a crucial part of treating bacterial infections. Unfortunately, bacteria are constantly evolving and becoming resistant to antibiotics.[6] Antibiotic resistance is when bacteria can resist the effects of the antibiotics, which makes them ineffective. This results from antibiotics being prescribed too frequently and unnecessarily. The bacteria are able to

mutate and create strains resistant to antibiotics. Antibiotic resistant bacteria cause 2.8 million infections in the United States and result in 35,900 deaths.[7]

One of the most common types of antibiotic-resistant bacteria is methicillin resistant *staphylococcus aureus* (MRSA). MRSA is likely to be transmitted in hospital environments by workers, patients, and equipment. MRSA strains carry the mec gene, which encodes for the protein PBP-2a (penicillin-binding protein 2a).[8] This protein allows the bacteria to create peptidoglycan to synthesize the cell wall even in the presence of antibiotics, because it doesn't need to bind to beta-lactams.[8]

Antibiotic resistance is occurring more frequently because of the over-prescription of existing antibiotics and the lack of production of new antibiotics. Antibiotics are often over-prescribed by healthcare providers. The over-prescription of antibiotics increases the chance that resistance will occur sooner. Therefore, pharmaceutical companies don't want to spend time and money creating new antibiotics for bacteria to become resistant to them a few years later.

1.3 Phospholipid Synthesis in S. aureus

Phospholipids are an important component of cell membranes. They provide structure to the cell, and changes in the composition of the membrane can impact processes such as antimicrobial resistance and other cellular processes.[9] These lipids contain glycerol with fatty acids that create the backbone, and phosphoric acid with an alcohol group. The alcohol group determines what category of phospholipid it is, phosphatidylglycerol (PG), diglycosyldiacylglycerols (DGDGs), or a Lysly-phosphatidylglycerols (Lysl-PGs). The acyl tails of phospholipids impact membrane fluidity. The acyl tails are composed of fatty acids, fatty acids are long carbon chains with a carboxyl group at one end. Fatty acids can be found in various locations throughout the cell. They play a crucial part in the phospholipid synthesis of *S.*

aureus. Saturated fatty acids have no double bonds between the carbon atoms, such as palmitic acid (PA) or stearic acid (SA).[10] Unsaturated fatty acids have at least one double bond between the carbon atoms, such as oleic acid (OA).[10] Saturated fatty acids produce acyl tails without kinks. This allows for the phospholipids to be tightly packed, creating a rigid membrane. Unsaturated fatty acids produce acyl tails with kinks. These kinks disrupt the tight packing of the membrane, which creates a more fluid membrane.

1.4 Antibiotics and Mechanisms of Action

Antibiotics can induce cell death through different mechanisms. Antibiotics can target the cell wall, DNA gyrase, folate synthesis, and RNA polymerase to induce cell death.[11] Penicillin is one of the most common antibiotics used to treat *S. aureus*. Penicillin is one of the most common antibiotics used to treat *S. aureus*. When penicillin was introduced, there was a significant improvement in the treatability of *S. aureus*.[11] Penicillin and other β -lactam antibiotics attack the cell wall of *S. aureus*. The β -lactam ring binds to the enzyme responsible for creating the cell wall in *S. aureus*.[12] This results in the disruption of the cell wall and causes the bacterial cells to die.[12]

Methicillin was the first antibiotic synthesized to be resistant to β -lactamase.[11] β-lactamase are enzymes that are produced by bacteria and cause resistance to β -lactam antibiotics. They break down the β -lactam ring, which causes them to be ineffective. Methicillin resists the destruction of the β -lactam ring, which allows the cell to continue to create a cell wall and prevent cell death.[11]

Quinolone antibiotics were originally created for infections caused by gram-negative bacteria. Soon after, they were used to treat gram-positive infections such as staphylococci.[11] Quinolones inhibit enzymes that are important for the process of DNA replication DNA gyrase

and topoisomerase IV.[11] These enzymes are responsible for unwinding DNA for replication.[11] Without the cells ability to replicate its DNA, it causes the cells to die. Examples of quinolones include ofloxacin, levofloxacin, and ciprofloxacin.

Vancomycin antibiotics were created to treat infections caused by gram-positive bacteria that are resistant to other antibiotics.[11] Vancomycin prevents the creation of peptidoglycans in the cell wall.[12] The cell wall is composed of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) glycan chains with cross-linked peptide chains. Vancomycin binds to D-alanyl D-alanine, which prevents the creation of the NAM and NAG glycan chains.[12] Without the ability to create a cell wall, the bacterial cells will eventually die.[12]

Daptomycin is a cyclic lipopeptide that treats different infections caused by gram positive bacteria.[13] Daptomycin is used as an alternative to vancomycin against MRSA and other gram-positive strains.[14] In *S. aureus*, daptomycin targets the cell membrane through a calcium dependent process. Daptomycin binds to the overall negatively charged portions of the membrane, for example, at the sites where phospholipid phosphatidylglycerol (PG) are present.[15] Daptomycin binds to the cell membrane in clusters, and the clusters integrate themselves into the membrane to create pores. These pores allow contents of the cell to leak out, which disrupts various processes within the cell, ultimately resulting in cell death.

1.5 Antibiotic Resistance in S. aureus

Penicillin resistant *S. aureus* strains were discovered in 1942, shortly after the creation of the antibiotic.[11] *S. aureus* that are resistant to penicillin is caused by the expression of the *blaZ* gene.[11] This gene encodes for β -lactamase, which hydrolyzes the ring on B-lactams.[11] This results in the B-lactams being inactive.

S. aureus that are methicillin resistance contain the mecA gene.[11] The mecA gene encodes for the penicillin-binding protein 2a (PBP2a). This protein allows for the bacteria to create peptidoglycan, which is the basic structure of the cell wall.[11] PBP2a inhibits the binding of all β -lactams but allows for the reaction necessary to produce peptidoglycan.[11] This mechanism allows for *S. aureus* to survive, even in the presence of antibiotics.[11]

S. aureus resistance to quinolone antibiotics is caused by mutations.[11] These mutations are often created by environments with high concentrations of bacteria and low concentrations of quinolones.[11] The amino acid changes that occur are in the quinolone resistant-determining region (QRDR).[11] These are regions of DNA gyrase and topoisomerase IV where the specific mutations occur. The most common mutations occur in the GrlA subunit of topoisomerase IV and the GryA subunit of DNA gyrase.[11] However, multiple mutations can occur within the QRDR, which increases S. *aureus* resistance to quinolones.[11]

S. aureus resistance to vancomycin is caused by the *vanA* operon. This allows the cell wall to modify the amino acid residues.[11] These modifications prevent vancomycin from binding to the cell wall. The cell wall synthesis continues, and *S. aureus* becomes resistant to the antibiotic as shown in Figure 1.1.[11]

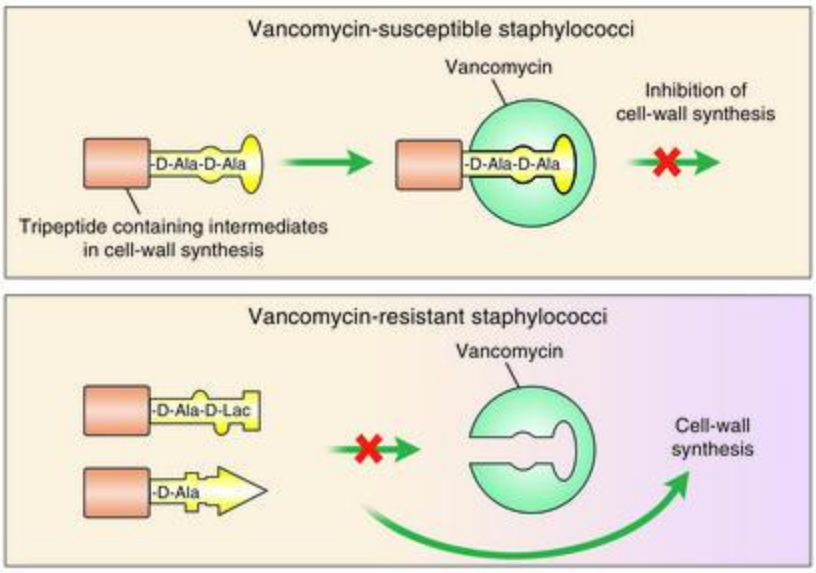

Figure 1.1. Mechanism of Resistance for *S. aureus* and Vancomycin.[11]

1.6 Proposed Mechanism for Daptomycin Resistance in S. aureus

Daptomycin resistance in *S. aureus* is uncommon, but not impossible.[13] Daptomycin resistance in *S. aureus* is normally associated with mutations in multiple peptide resistance factor (mprF), CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (*pgsA*), and cardiolipin synthase (cls2) as shown in Figure 1.2.[16] *PgsA* is responsible for the production of the PG by converting CDP-diacylglycerol (CDP-DG) to PG. Mutations in *pgsA* result in a decrease in production of PG.[16] This causes a decrease in the overall charge of the membrane.[16] This will result in the repulsion of daptomycin, because daptomycin wants to bind to the negatively charged portions of the membrane. MprF is the gene responsible for the lysylation of PG, which results in an L-PG. The headgroup of the L-PG has an overall positive charge. When integrated into the membrane, it results in an overall increase in the positive charge. The mutation of mprF will cause over-production in L-PG, which will increase the overall charge of the membrane to positive.[16] This will result in the repulsion of daptomycin. Cardiolipin synthase (*cls2*) is a gene that encodes for a protein in phospholipid synthesis. Cardiolipins are synthesized by an enzyme

in the membrane, Cls. The mutation of the *cls2* gene decreases the function of Cls.[16] This results in an overall decrease in the negative charge of the membrane, which will result in the repulsion of daptomycin.[16]

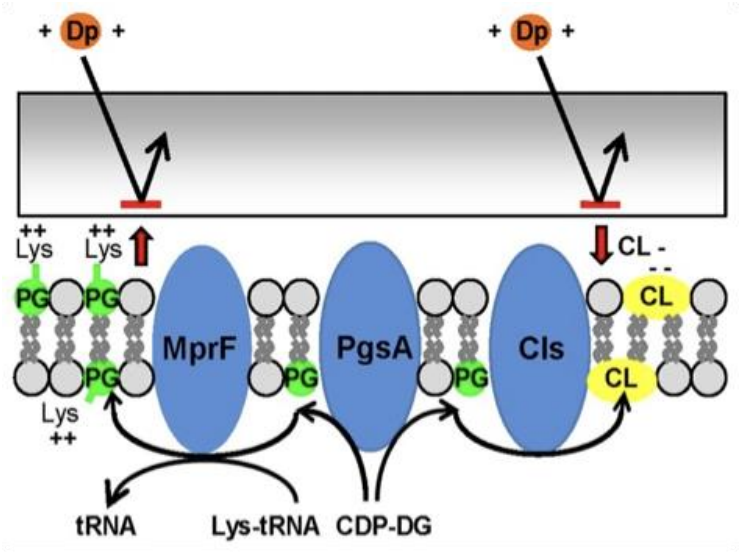

Figure 1.2. Proposed Mechanism of Daptomycin Resistance.[16]

1.7 Phospholipid Synthesis in S. aureus

Phosphatidic acid (PA) is the main precursor in the synthesis of phospholipids. PA is synthesized through a two-step acylation reaction. This reaction involves the acyl-ACP (acyl carrier protein) transferases using the *PlsX/PlsY/PlsC* enzyme pathway. Acyl-ACP is converted to acyl-phosphate (acyl-PO_4) by *PlsX*. Acy- PO_4 catalyzes the acylation of glycerol-3-phosphate (G3P) to 1-acyl-G3P using the PlsY enzyme pathway. PlsC transfers a fatty acid to acyl-G3P. This creates a phosphatidic acid, the precursor.[9] CDP-diacylglycerol (CDP-DAG) is synthesized from phosphatidic acid and cytidine triphosphate (CTP). The CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase (*pgsA*) assists in glycerolphosphate replacing a cytidine monophosphate.[9] This step generates a phosphatidylglycerolphosphate (PG-P). PG-P is

dephosphorylated to create a PG.[9] PG can be synthesized by *cls1* and *cls2* to produce cardiolipin. PG can be aminocylated to produce a lysyl-phosphatidylglycerol.[9]

1.8 Fatty acid Biosynthesis in S. aureus

Fatty acid synthesis in *S. aureus* used fatty acid synthesis type II (FASII) process. This pathway involves the use of host derived fatty acids. FASII involves a lot of energy and is comprised of two important steps, initiation and elongation.[17] Initiation begins with acetyl-CoA carboxylase (AccABCD) adding a carboxyl to acetyl-CoA to form malonyl-CoA. Manoyl-CoA is converted to manoyl-ACP by malonyl-CoA:ACP transacylase (FabD).[17] β-Ketoacyl-ACP synthase III (FabH) combines malonyl-ACP with acetyl-CoA to create acetoacetyl-ACP.[17] This reaction begins the elongation process. The acyl chain is extended by β-ketoacyl synthase I or II (FabB or FabF). This results in an acyl chain that is two carbons longer.[17] This process can continue for multiple carbon extensions. NAD(P)H-dependent enoyl-ACP reductase (FabI) catalyzes the last step of fatty acid synthesis by forming the desired length acyl-ACP.[18]

This process is so essential in *S. aureus*, that drugs are often synthesized to disrupt this pathway. For example, AFN-1252 is an inhibitor of FabI in *S. aureus*, as shown in Figure 1.3.

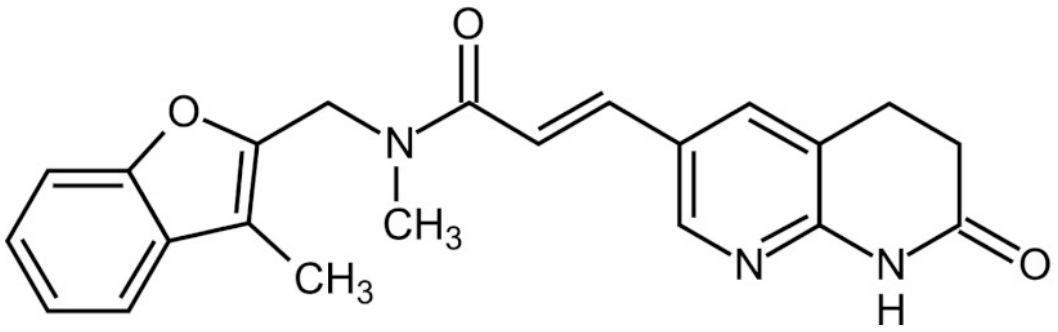

Figure 1.3. Structure of Abafacin desphosphono AFN-1252.[19]

FabI performs the final step in fatty acid biosynthesis pathway, it is responsible for elongating the fatty acid.[19] This disruption impacts the saturated and unsaturated fatty acid biosynthesis pathway and will prevent bacterial growth.[19] Enoyl-acyl carrier protein (Enoyl-ACP) has four

enzyme forms: FabI, FabK, FabL, and FabV. FabI is only present in *S. aureus*, therefore, it is essential to cell viability in the Staphylococcus spp.[20]

1.9 fakA::Tn and the Incorporation of Straight Chain Fatty Acids

Gram positive bacteria use the fatty acid kinase (fak) complex to gather and phosphorylate fatty acids to prepare for phospholipid synthesis as shown in Figure 1.4.[22] The complex has two important components, fakA and fakB. fakB is a carrier protein that transports a fatty acid to fakA for phosphorylation as .[22] fakB has two components, fakB1 and fakB2. fakB1 interacts and incorporates saturated fatty acids.[22] fakB2 only interacts with unsaturated fatty acids, but *S. aureus* doesn't incorporate unsaturated fatty acids.[22] fakA is responsible for the phosphorylation and incorporation of exogeneous fatty acids into the lipid membrane.[22]

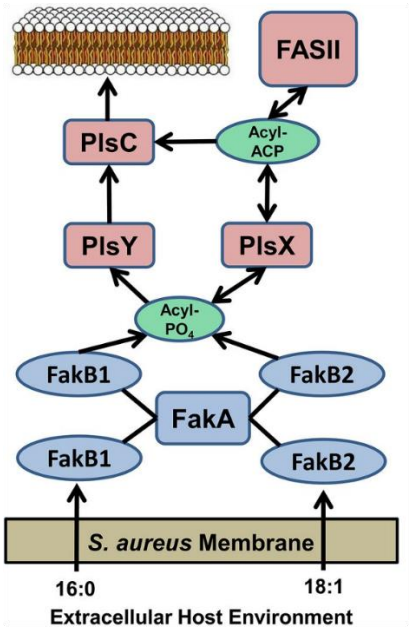

Figure 1.4. Fatty Acid Kinase Dependent Exogenous Fatty Acid Uptake.[21]

1.10 Incorporation of OA into PG

S. aureus naturally produces saturated PGs (2).[23] Unsaturated PGs are present in *S. aureus* because of the supplementation of exogenous fatty acids and the presence of fakA. Previous work in the Hines lab is shown in Figure 1.5, which shows different strains of *S. aureus*

supplemented with OA. Saturated fatty acids are present in each of the strains and conditions. There are only unsaturated fatty acids present in the conditions supplemented with OA and the strains that contain fakA. fakA phosphorylates the exogenous OA, which is elongated by the FASII pathway, and incorporated into the membrane as a PGs. Since fakA is only present in the JE2 and Dap2 strains, there is only unsaturated fatty acids present in those strains with OA supplementation.

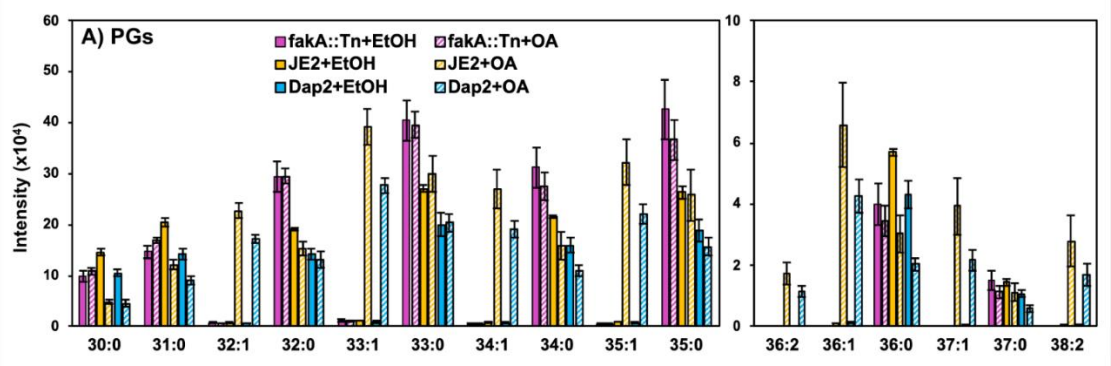

Figure 1.5. The PGs in JE2, *fakA::Tn*, and Dap2 with the supplementation of OA.

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CHAPTER 2

OPTIMIZATION OF S. AUREUS BIOFILM GROWTH AND QUANTIFICATION

1.0 Introduction

1.1 Bacterial Biofilms

Bacterial biofilms are microbial communities that are enclosed in polysaccharides, proteins, lipids, and DNA produced by the bacteria.[1] These components, which make up the extracellular polymetric substances (EPS), protect and provide structure to the biofilm.[1] The creation of biofilms is caused by cells aggregating or adhering to surfaces.[1] Due to bacteria's ability to attach to a surface and rapidly grow, biofilm bacterial infections can be frequent. The biofilm acts as a protective barrier over the bacteria, making it hard to treat the infections.[1] The bacteria can latch onto skin and a variety of surfaces, such as medical devices, contributing to the increase of bacterial infections in hospital settings.[1]

1.2 Bacterial Biofilm Formation

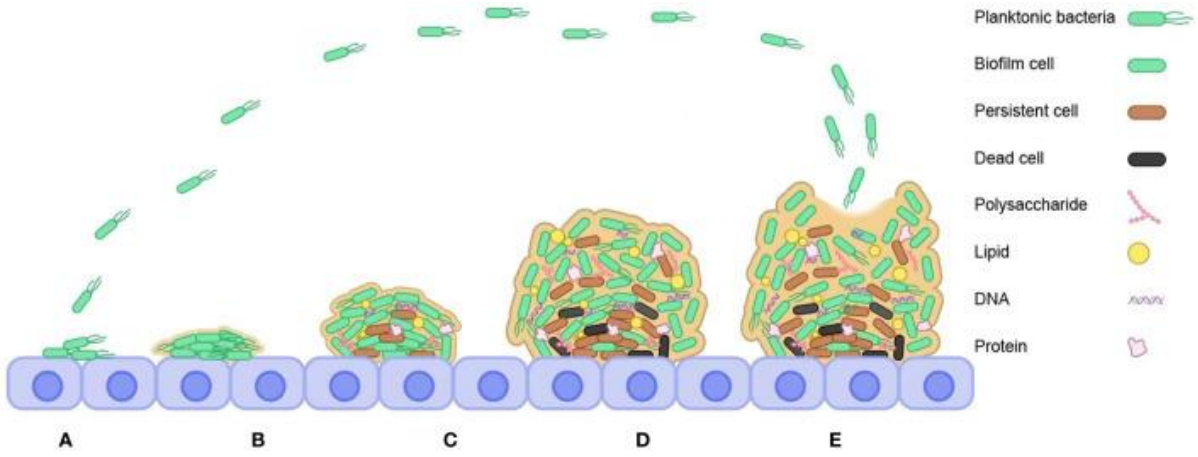

Figure 1.1. Step by Step Process of Biofilm Formation.[1]

The formation of biofilms is a process that involves multiple steps which includes reversible attachment, irreversible attachment, maturation, and dispersal.[1] As seen in Figure 1.1, the cells begin the biofilm formation process by attaching to a surface such as medical devices, aquatic systems, and the human body. Once the bacteria begin to grow, chemotaxis allows the bacteria to find nutrient sources and strongly interact with the area of attachment.[1] However, this is still considered a reversible attachment. Now that the cells have attached, flagella and fimbriae begin to form the biofilm. Flagella are responsible for commencing the bacterial cells to adhere to the area of attachment.[1] The bacteria begin to produce the extracellular polysaccharide matrix EPS, which provides structure for the biofilm to properly grow and develop.[1] The formation of the EPS protects the bacteria and indicates irreversible attachment. The bacteria begin to mature and produce colonies within the EPS.[1] Once the bacteria matured, cells detach from the colonies that formed, and those cells are dispersed. The dispersed cells can begin to attach to other cells or another surfaces, and then the process starts from the beginning.[1]

1.3 Palmitic Acid and Oleic acid

Palmitic acid (16:0), as shown in Figure 1.2, is a common saturated fatty acid that can be found in multiple types of food, and one of the most common fatty acids in the human body.[2] Palmitic acid is highly concentrated in membrane phospholipids and adipose triglycerides.[2] The incorporation of palmitic acid into the membrane as a phospholipid and as a free fatty acid results in a rigid membrane. Rigid membranes increase adhesion in bacterial cells, which can result in an increase in biofilm formation.[3] Oleic acid (18:1), as shown in Figure 1.2, is a common unsaturated fatty acid that can be found in oils, nuts, and meat products. Oleic acid has been observed to aid in daptomycin resistance. Therefore, it could have interesting impacts on biofilm production. The incorporation of oleic acid into the membrane as a phospholipid and as a

free fatty acid result in a more fluid membrane due to the unsaturation creating kinks in the structure of the FA.[3]

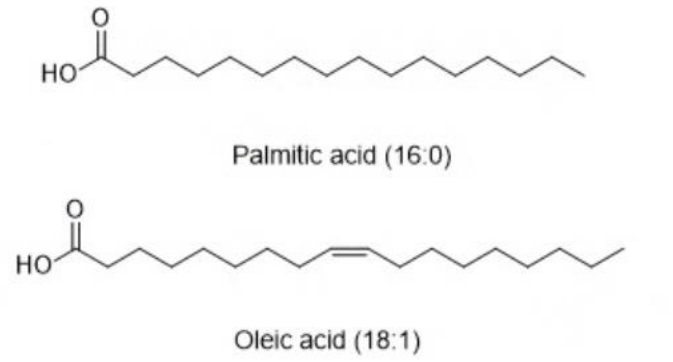

Figure 1.2. Structures of Palmitic acid and Oleic acid.

2.0 Methods

2.1 Determining Ideal Concentration of Fatty Acid to Optimize Biofilm Growth: Protocol #1

S. aureus JE2 was streaked on tryptic soy agar (TSA) and grown statically overnight at 37°C. The bacterial suspension was made to an optical density of $6.0X10^8$CFU/mL or 2 McFarland in sterile 0.9% w/v sodium chloride. 100 µL of bacterial suspension was added to tryptic soy broth (TSB) with a fatty acid and a control corresponding to the fatty acid storage condition. The plate was sealed and incubated at 37 °C for 24 hours, with no shaking. The medium was removed without disrupting the biofilm. The biofilm was gently but rapidly washed with 1 mL of 10x PBS. The biofilm was dried until it was no longer milky-white. 500 µL of 0.1% safranin dissolved in 50% ethanol was added to each well for 15 minutes. The safranin was removed, and each well was washed with 1 mL of 10x PBS. The safranin was eluted with 70/10/20 EtOH/IPA/H_2O. After 15 minutes, each well was mixed, and the biofilm was scratched off the bottom of the well with a pipette tip. The de-stain removes the stain from the biofilm, but the biofilm still remains at the bottom of the well. The biofilm was scratched off the bottom of

the well to avoid impacting the absorbance readings. The absorbance (λ = 530 nm) of each well was measured. This protocol was obtained from the Peschel lab at the German Center for Infection Research (DZIF).[4] In a 24-well plate, 890 µL of TSB, 100 µL of bacterial suspension, and 10 µL of 5 mM, 10 mM, and 15 mM PA and OA were added to wells in triplicate. The $O.D._{530}$ readings were obtained to quantify the amount of biofilm produced as shown in Figure 2.1.

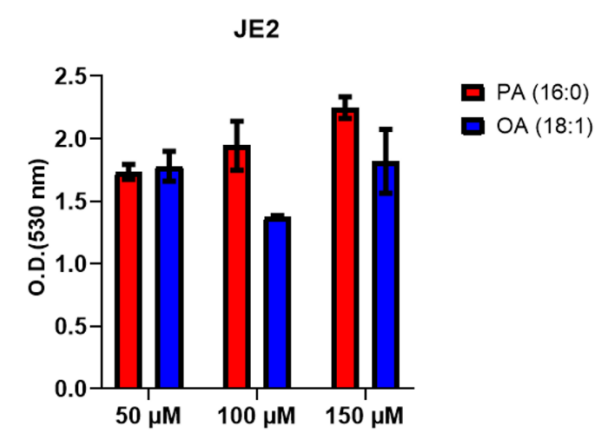

Figure 2.1. *S. aureus* JE2 Growth in the Presence of Palmitic Acid and Oleic Acid.

The wells containing 150 µM PA had the highest OD reading and therefore, the most biofilm growth compared to the other experimental concentrations. The wells containing 150 µM OA had a higher OD than the 100 µM trial, though the OD and biofilm growth was not statistically different than the 50 µM for the OA wells. Due to the collective performance of the FAs at 150 µM, that concentration was selected for the remainder of the biofilm experiments.

2.2 Incorporating the Centrifuge to Prevent Loss of Biofilm: Protocol #2

While conducting these initial experiments, the biofilm was thin and difficult to work with. It was difficult to wash the biofilm with 10x PBS without tearing the biofilm within the well, which causes the $O.D._{530}$ readings to not accurately represent the amount of biofilm that

grew in the wells. The protocol was altered to incorporate the centrifuge. Centrifuging the 24 well-plates, keeps the biofilm at the bottom of the well while it is washed with 10x PBS. The plate was sealed and incubated at 37 °C for 24 hours, with no shaking. The medium was removed without disrupting the biofilm. Instead of washing the biofilm with 1 mL of 10x PBS, PBS was added to the well and mixed to dissolve the biofilm. The plate was centrifuged at 2000 g for 10 minutes. The supernatant was removed, the biofilm was stained with 500 µL of 0.1% safranin dissolved in 50% ethanol for 15 minutes. The safranin was removed, 10x PBS was added to the well to dissolve the biofilm. The plate was centrifuged at 2000 rpm for 10 minutes. The supernatant was removed, de-stain was added, the plate incubated at room temperature for 15 minutes. $O.D._{530}$ readings were collected from the 24 well-plate. 100 µL from each well was transferred to a 96-well plate for an $O.D._{530}$ reading. These changes to the protocol prevented the biofilm from tearing during the wash steps and impacting the $O.D._{530}$ readings.

An experiment was conducted with *S. aureus* JE2 with a final concentration of 150 µM PA on two identical plates to compare protocol #1 and protocol #2. The experiment was conducted in a 24 well-plate and then transferred to a 96 well-plate to obtain the $O.D._{530}$ readings to avoid scratching the biofilm off the bottom of each well. 200 µL of each well was transferred to a 96 well-plate. An additional 100 µL of each well was transferred to another 96 well-plates for the $O.D._{530}$ reading.

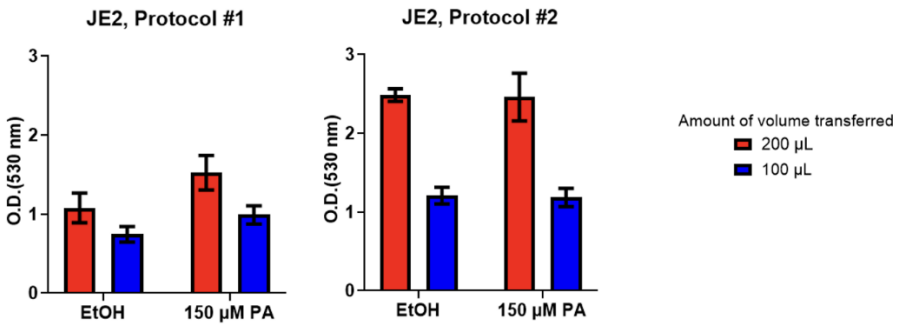

Figure 2.2. *S. aureus* JE2 Growth with 150 µM PA and EtOH: Comparing Protocol #1 and Protocol #2.

As shown in the OD readings in Figure 2.2, there is more biofilm growth using protocol #2 compared to protocol #1. The addition of the centrifuge prevented biofilm from being lost in the wash steps. The centrifuge helps the biofilm stay on the bottom of the well. When the biofilm is washed with PBS, it stays on the bottom of the well instead of detaching from the well. If detached from the well, the biofilm can be removed from the well with the PBS. Additionally, there is more error associated with the 200 µL transfer than the 100 µL transfer in protocol #2, particularly in the PA condition. Based on these results the protocol was modified to incorporate the changes with the centrifuge and the 100 µL transfer to a new well-plate.

2.3 High Variability from Residual Staining of Plate

While conducting experiments with protocol #2, there was still stain remaining in the well after washing the biofilm with PBS. As shown in Figure 2.3, the $O.D._{530}$ readings have a high variability because of the 0.1% safranin remaining in the well after the washing steps while following the steps of protocol #2.

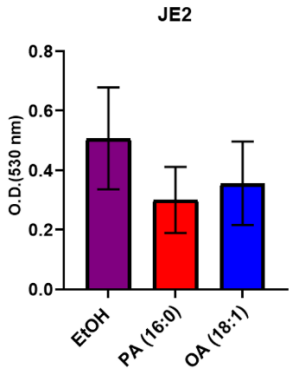

Figure 2.3. *S. aureus* JE2 Supplemented with PA, OA, EtOH, and Stained with 0.1% Safranin.

To combat this issue, I began staining the biofilm with 0.41% crystal violet instead of 0.1% safranin, while keeping all other steps of protocol #2 the same, to see if there was a decreased amount of leftover stain in the well. However, the $O.D._{595}$ readings still have high variability with the alternate stain as shown in Figure 2.4.

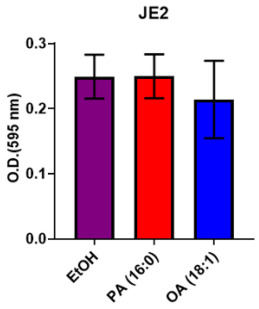

Figure 2.4. *S. aureus* JE2 Supplemented with PA, OA, EtOH, and Stained with 0.41% Crystal Violet

2.4 Biofilm Quantification: Protocol #3

This protocol was obtained from the Endres lab at the University of Nebraska Medical Center. The bacteria of choice were plated on TSA and grown statically for 24 hours at 37°C. The bacterial suspension was made in TSB supplemented with 0.5% Glucose and 3% NaCl. The suspension was diluted to an $O.D._{600}$ of 0.05. Each condition was pipetted into a 96 well-plate

and grown statically for 24 hours at 37°C. The plate was washed gently twice with 200 µL of 10x PBS. 100 µL of 100% ethanol was added to each well and incubated at room temperature for 2 minutes. 100 µL of 0.41% crystal violet in 50% ethanol was added to each well for 2 minutes. The liquid was removed, and the plate was washed three times with 200 µL of 10x PBS. 100% ethanol was added to each well to de-stain for 10 minutes. 50 µL of the solution from each well was transferred to another 96 well-plate to obtain an $O.D._{655}$ reading.

After conducting multiple trials, there was still 0.41% crystal violet in 50% ethanol that was remaining in the wells. It was also difficult to remove all the stain from the biofilm to obtain an accurate $O.D._{655}$ reading. Therefore, instead of using crystal violet, 0.1% safranin was used to stain the biofilm. The 0.1% safranin was removed from the sides of the wells, but there was still stain remaining in the biofilm. When 70/10/20 EtOH/IPA/H_2O was used instead of 100% ethanol, the stain was able to be removed from the biofilm.

2.5 Finalized Biofilm Quantification Protocol

S. aureus JE2 and *fakA::Tn* were streaked on TSA and grown statically for 24 hours at 37°C. Bacterial was suspended in TSB with 3% NaCl and 0.5% Glucose and then diluted to an $O.D._{600}$ of 0.05 in a 96 well-plate. In a 15 mL tube, a 5 mL solution was created with TSB, bacterial suspension, and fatty acids. Each tube contains a different fatty acid or control. The TSB and bacterial suspension were added to obtain a final $O.D._{600}$ reading of 0.05. OA and PA were added to obtain a final concentration of 150 µM.

The solution in the tubes was inverted to mix and 200 µL of each sample was pipetted into a 96 well-plate. The plate was sealed and incubated at 37 °C for 24 hours, with no shaking. The medium was removed without disrupting the biofilm. The biofilm was gently but rapidly washed twice with 200 µL of 10x PBS. Each well was stained with 50 µL of 0.1% safranin in

50% EtOH for about 15 minutes, ensuring the biofilm has absorbed the stain. The 0.1% safranin was removed, and each well was washed three times with 200 µL of 10x PBS. The safranin was eluted with 150 µL of 70/10/20 EtOH/IPA/H_2O for 1 hour on a rotating platform or until the 0.1% safranin is eluted from the biofilm. Transfer 100 µL from each well into a new 96 well-plate. The absorbance (λ = 530 nm) of each well was measured.

2.6 Comparing Data from Previous Protocols to Finalized Protocol

The incorporation of the TSB (Glu + NaCl) and the multiple washing steps promoted biofilm growth and decreased the variability within the $O.D._{530}$ readings. Figure 2.5 shows the same experiment conducted using protocol #1 on two separate days. There is variability between the $O.D._{530}$ readings obtained on different days from the same concentration of fatty acid. There is also variability between the $O.D._{530}$ readings obtained on the same day at the same concentration. Figure 2.6 is results from a comparison conducted according to the finalized protocol. *S. aureus* JE2 was supplemented with TSB (Glu + NaCl) or TSB, PA, OA, and EtOH. The results are an average of 3-inter day trials. The results in Figure 2.6 have $O.D._{530}$ readings that are significantly higher than the readings in Figure 2.5. Supplementing TSB with NaCl and Glucose significantly increased biofilm production. There is less variability in the results in Figure 2.6 compared to Figure 2.5. Based on the increase in $O.D._{530}$ readings with a decrease in variability between the readings, the finalized protocol was implemented.

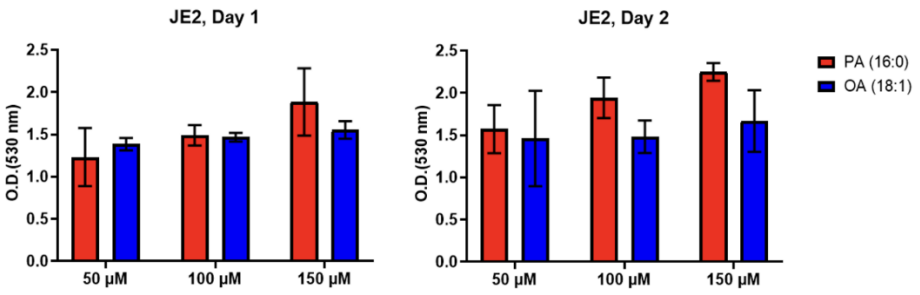

Figure 2.5. Biofilms of *S. aureus* JE2 Supplemented with PA and OA using protocol #1.

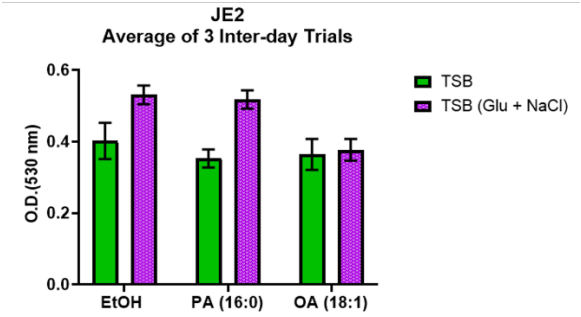

Figure 2.6. Biofilms of *S. aureus* Supplemented with PA, OA, and EtOH using Finalized Protocol.

3.0 Results and Discussion

3.1 S. aureus JE2 fakA::Tn Growth Comparison

Using the finalized protocol, *S. aureus* JE2 and *fakA::Tn* were grown in the presence of PA, OA, and EtOH. The results, as shown in Figure 3.1, are an average of 3 Inter-day Trials. These results differ from the expected trend. JE2 contains fakA, which is responsible for the phospholipid synthesis by incorporating fatty acids into the lipid membrane of *S. aureus.*[5] The incorporation of PA results in a more rigid membrane, which promotes adhesion and increases biofilm formation. *S. aureus fakA::Tn* doesn't contain fakA, therefore PA can't be incorporated into the membrane. Therefore, biofilm formation isn't promoted. The mutation of fakA can cause an increase in the production of SdrD adhesion, which increases biofilm production.[6] Serine Aspartate repeat containing protein D (SdrD) increases biofilm formation because it is responsible for bacterial adhesion and colonization.[7] The presence of free fatty acids in the membrane of *S. aureus fakA::Tn* could also cause an increase in biofilm formation.

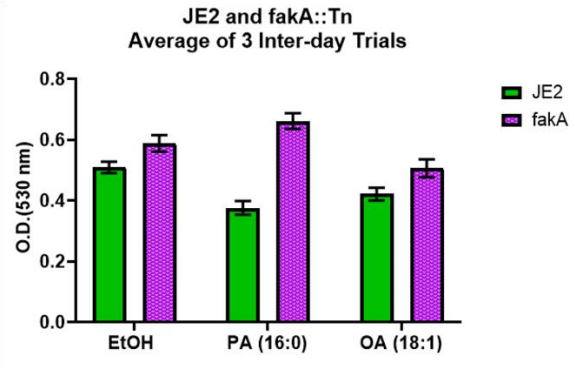

Figure 3.1. *S. aureus* JE2 and fakA::Tn Growth in the Presence of PA, OA, and EtOH using Finalized Protocol.

3.2 S. aureus JE2 and fakA::Tn Mutant Growth Comparison in the Presence of AFN-1252

Using the finalized protocol, *S. aureus* JE2 and *fakA::Tn* mutant was grown in the presence of PA, OA, DMSO, with the supplementation of AFN-1252. The results, as shown in Figure 3.2, are an average of 3 Inter-day Trials. *S. aureus* JE2 produces more biofilm because of the presence of fakA. fakA phosphorylates PA and OA and incorporates them into the membrane. The incorporation of PA promotes biofilm formation, and the incorporation of OA inhibits biofilm formation. The lack of incorporation of exogenous fatty acids and the disruption of the endogenous fatty acid synthesis by AFN-1252 results in a lack of biofilm formation. AFN-1252 targets FabI, an enzyme that is important in fatty acid synthesis, in *S. aureus*.[8] FabI is responsible for the last step of the fatty acid synthesis pathway.[9] This disruption can decrease the amount of biofilm production. Therefore, the combination of the *S. aureus fakA::Tn* mutant and AFN-1252 decreased biofilm formation compared to the *S. aureus* JE2 strain.

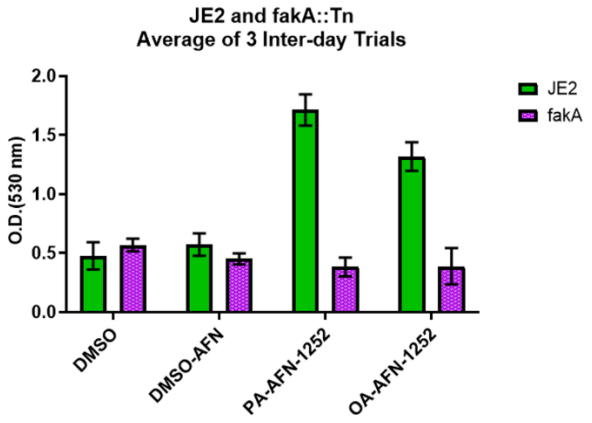

Figure 3.2. *S. aureus* JE2 and fakA::Tn Growth in the Presence of PA, OA, EtOH and AFN-1252.

4.0 Conclusions

In conclusion, I finalized a method for quantifying biofilm growth in *S. aureus*. Using this method, I compared *S. aureus* JE2 and *fakA::Tn* mutant with the supplementation of PA, OA, and EtOH. *S. aureus fakA::Tn* mutant produced more biofilm than *S. aureus* JE2. These results were different than the expected trend, but the overproduction of SdrD could result in more biofilm formation. I compared *S. aureus* JE2 and *fakA::Tn* mutant grown in the presence of PA, OA, and EtOH with the supplementation of AFN-1252. JE2 *S. aureus* with PA and OA produced more biofilm than *S. aureus fakA::Tn* mutant. This trend is expected because the *S. aureus fakA::Tn* can't incorporate PA and OA into the membrane. AFN-1252 is targeting the FASII pathway and disrupting the endogenous FA synthesis. These factors make it difficult for the *fakA::Tn* to produce biofilm.

5.0 References

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CHAPTER 3

IMPACT OF FATTY ACIDS AND ANTIBIOTICS ON THE GROWTH OF STAPHYLOCOCCUS AUREUS

1.0 Introduction

1.1 Bacterial growth cycle

Bacteria that grow in a contained system grow in four phases as shown in figure 1.1.[1] The lag phase is when cells have been re-located to their new environment and are adapting.[1] The cells are growing in an environment with broth that contains nutrients that are necessary for cell growth. As the bacteria adjust to their new environment, the bacterial cells prepare for division. They often increase in size, but they aren't dividing yet. The log phase begins when the cells are dividing and exponentially growing.[1] The cells are dividing by binary fission, which means they are doubling each generation. The significant amount of cell growth creates an exponential curve. The stationary phase begins when the rate of cell growth is equivalent to the rate of cell death.[1] The nutrients for the cells are limited, and the conditions are no longer ideal for reproduction. The death phase begins when the rate of cell death is greater than the rate of cell growth.[1] The environment has a buildup of waste and cells are no longer maintaining metabolic functions.

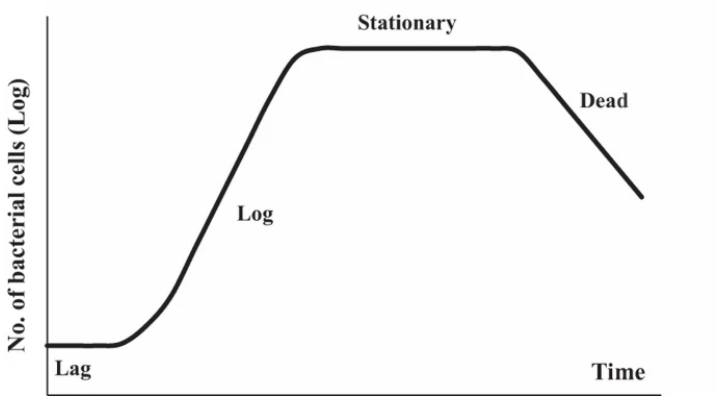

Figure 1.1. Growth Pattern of Bacteria in a Contained System.[1]

1.2 Antibiotics and the Impact on the Growth Cycle

Growth curves provide the opportunity to view the impact of antibiotics on bacterial cell growth. Antibiotics induce cell death through a few different pathways. Antibiotics can attack a specific system in the cell.[2] This attack will either cause the cells to die or just prevent them from growing.[2] *S. aureus* JE2 and fakA::Tn mutant are susceptible to daptomycin. Daptomycin binds to the overall negatively charged portions of the membrane, for example, at the sites where phospholipid phosphatidylglycerol (PG) are present.[3] The daptomycin binds to the cell membrane in clusters, and the clusters integrate themselves into the membrane to create pores. These pores allow contents of the cell to leak out, which disrupts various processes within the cell, ultimately resulting in cell death. The impact daptomycin has on the growth of *S. aureus* can be observed through growth curves.

1.3 OA Incorporation in S. aureus JE2 and fakA::Tn and the Impact on Daptomycin Resistance

Previous work in the Hines lab evaluated the impact of OA on daptomycin resistance in *S. aureus* JE2 and *fakA::Tn*. Figure 1.2 shows the growth cycle of JE2 and fakA::Tn. JE2 contains fakA::Tn, which allows the cells to incorporate the exogenous OA into the membrane as a phospholipid. The *fakA::Tn* mutant doesn't contain *fakA::Tn*, so this strain can only

incorporate OA as free fatty acids. As shown in Figure 1.2, oleic acid promotes daptomycin resistance in both strains. In Figure 1.2, the samples supplemented with EtOH and daptomycin have little or no growth. The samples supplemented with OA and daptomycin, the strains continue to grow, though to a lesser degree and at a slower rate than without the challenge of daptomycin. Therefore, OA promotes daptomycin resistance when incorporated as free fatty acids or fatty acyl tails.

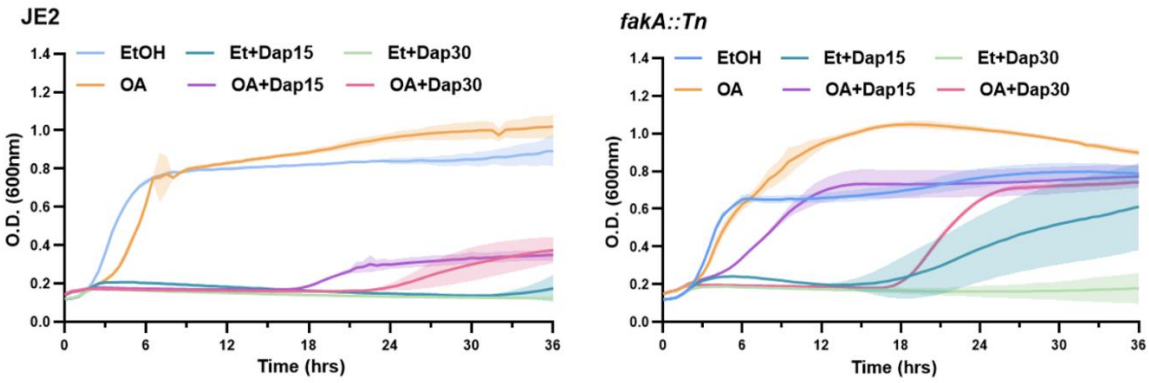

Figure 1.2. Incorporation of OA and Daptomycin in *S. aureus* JE2 and fakA::Tn.

1.4 OA is Hydrated by Oleate Hydratase to Produce 10-Hydroxystearic Acid (10-HSA)

Oleate hydratase (OhyA) is an enzyme that catalyzes the addition of water to the double bond of oleic acid.[4] This reaction produces 10-Hydroxystearic acid (10-HSA) and so the presence of 10-HSA can confirm oleic acid incorporation in the membrane. *S. aureus* JE2, *fakA::Tn*, and Dap2 were supplemented with OA and EtOH to assess the 10-HSA presence in the strains following OA supplementation. Previous work in the Hines lab is displayed in Figure 1.3, which shows the presence of OA and 10-HSA in the samples where JE2, *fakA::Tn*, and Dap2 were supplemented with OA. OA and 10-HSA are not present in the samples supplemented with EtOH while FA 20:1 and FA 22:1 is present in the samples where JE2 and Dap2 were supplemented with OA. JE2 and Dap2 contain *fakA::Tn*, which allows them to phosphorylate the

exogeneous OA and integrate it into the membrane as a phospholipid. After phosphorylation, the FASII pathway can elongate. OA (18:1) to FA 20:1 and FA 22:1. These fatty acids are not present in *S. aureus* fakA::Tn mutant because OA can't be elongated without the presence of fakA::Tn.

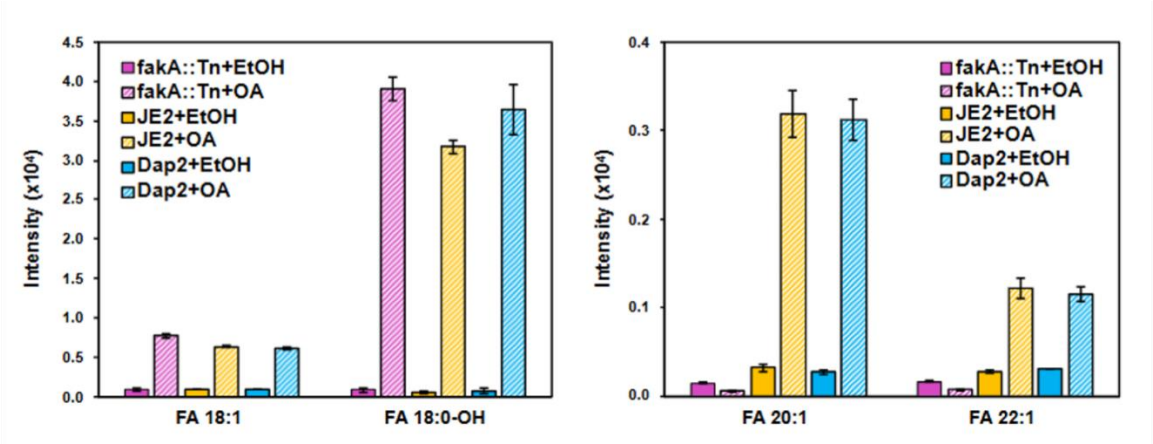

Figure 1.3. Incorporation of OA in *S. aureus* fakA::Tn, JE2, and Dap2.

1.5 Impact of OA derivatives on S. aureus JE2 and fakA::Tn Mutant

Based on the results in Figure 1.2, the presence and integration of OA in the membrane promotes daptomycin resistance. OA is hydrated by OhyA to produce 10-HSA. The increased resistance to daptomycin could be from the OA or the 10-HSA. OhyA only reacts with OA, the impact of structurally similar FA on daptomycin resistance remains to be determined. Figure 1.4 shows the FA selected to determine the impact of structural differences on daptomycin resistance. *Cis*-vaccenic acid (*cis*-VA, 18:1) is a FA with 18 carbon atoms and 1 double bond, it is a structural isomer of oleic acid. The double bond location on *cis*-VA is on carbon 11 and OA is on carbon 9, as shown in Figure 1.4. *cis*-VA will help determine if the location of the double bond impacts the increased resistance to daptomycin. *Cis*-9,10-methyleneoctadecanoic acid (*cis*-MOA) is a fatty acid with 19 carbons with a cyclopropane. *Cis*-MOA will help determine if the

presence of a double bond is necessary for daptomycin resistance, or if the presence of a cyclopropane impacts daptomycin resistance.

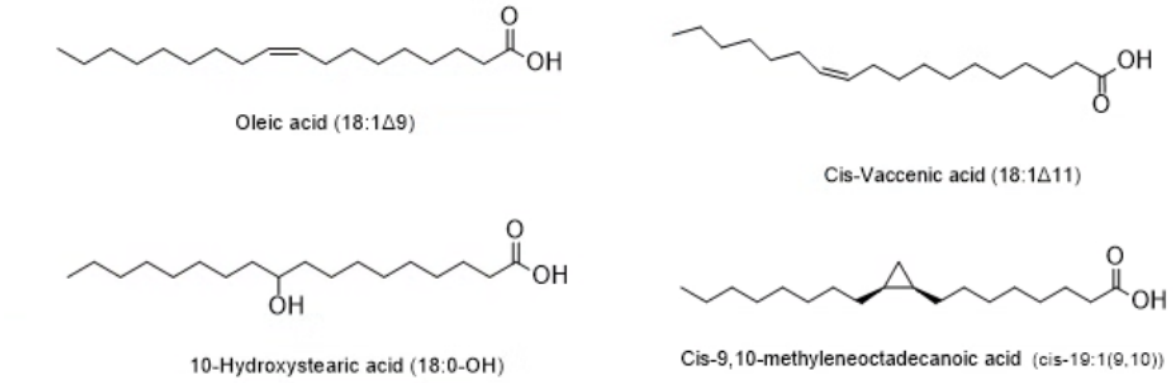

Figure 1.4 Structures of OA, 10-HSA, *cis*-VA, and *cis*-9,10-MOA.

2.0 Methodology

2.1 Lipid Extraction

For all LC-MS experiments, 2 mL of 2 McFarland (600 nm) suspensions of *S. aureus* JE2 were cultured in tryptic soy broth (TSB) and ethanol, OA, *cis*-VA, *cis*-9,10-MOA, 10-HSA, so that the total concentration of exogenous FA was 100 µM. The bacteria grew overnight, in triplicate, in an incubator shaker at 37°C and 200 rpm. The pellet was washed in 2 mL of sterile water and the optical densities (OD_{600nm}) of each sample was measured. Bacteria pellets were then extracted using a modified version of the Bligh & Dyer method.[5, 6] The pellets were washed and resuspended in 0.5 mL of HPLC grade water, sonicated, and then 2 mL of chilled 1:2 chloroform/methanol was added. After vortexing periodically for 5 min, 0.5 mL of chilled chloroform and water were added to induce phase separation. The samples were briefly vortexed and centrifuged for 10 minutes. The lower organic layer was collected, dried under vacuum, and reconstituted in 1:1 chloroform/methanol.

2.2 LC-MS

Lipid extracts and a quality control (QC) pooled mixture of 5 µL of each sample samples were analyzed using a Waters Acquity FTN I-Class Plus ultra-performance liquid chromatography (UPLC) system equipped with a Waters CORTECS HILIC (2.1x100 mm, 1.6 µm) column for hydrophilic interaction chromatographic separation. Lipid extracts were prepared at a 300x dilution. For HILIC, MPA consisted of 95:5 acetonitrile/water with 10 mM ammonium acetate and MPB consisted of 50:50 acetonitrile/water with 10 mM ammonium acetate. A 7 min gradient at a 0.5 mL/min flow rate was performed with the following conditions: 0-0.5 min, 100% MPB; 0.5-5 min, 100-60% MPB; 5-5.5 min, 60% MPB; 5.5-6 min, 60-100% MPB; 6-7 min, 100% MPB.

The column temperature was kept at 40°C and 5 µL injection volume, maintained at 6°C in the autosampler, was used for each sample. The Waters Acquity UPLC was connected to the electrospray ionization source of a Waters Synapt XS traveling-wave ion mobility mass spectrometer (TWIM-MS). Traveling wave separations were done with a wave velocity of 550 m/s and a wave height of 40 V with a nitrogen flow of 90mL/min. Mass calibration was performed with sodium formate over a 50-1200 *m/z* mass range. The samples were analyzed in the negative ionization mode. For HILIC, data was collected over the 7 min with a collision energy ramp of 40-60 eV.

2.3 Data Analysis

Progenesis QI (v3.0, Waters/Nonlinear Dynamics) was used to analyze the Waters.raw files with lock-mass correction and align the samples with a quality control reference sample. Peak picking was performed, and the data was normalized to the reference sample in Progenesis. The abundance of the peak areas of the PG precursors were calculated in Progenesis and

exported to Excel. PGs were evaluated as [M-H]- adducts. All lipid precursors were identified using the database LipidPioneer[7] with an accurate mass (< 4 ppm tolerance).

3.0 Methodology

3.1 Growth curve Lag Phase Extension Assay

S. aureus JE2 and *fakA::Tn* were plated on TSA and grown at 37°C, statically for 24 hours. Bacterial suspensions were made with $6.0X10^{8}$CFU/mL or 2 McFarland in sterile 0.9% w/v sodium chloride. In a 15 mL tube, 500 µL of the suspension was added to TSB with 100 µM fatty acid or ethanol. The samples were incubated overnight on an incubator shaker at 37°C. The following day, the broth was discarded after the samples were centrifuged at 2000 g for 10 minutes, and the pellet was re-suspended in sterile 0.9% w/v sodium chloride. The pellet was adjusted to 2 McFarland in sterile 0.9% w/v sodium chloride. TSB was divided into three tubes per condition per fatty acid and ethanol. TSB with 30 mg/mL calcium chloride was used to overcome the charge-charge repulsion between daptomycin and phospholipids. 2 McFarland suspension was added to each tube to obtain a final $O.D._{600}$ reading of 0.05. For each strain and experimental condition, one tube contains just the fatty acid, one contains fatty acid with 15 ug/mL daptomycin, and one contains fatty acid with 30 ug/mL daptomycin. The tubes were mixed and then added into a row on a 96 well-plate, with 5 replicates for each condition. The plate was placed in a plate reader to obtain $O.D._{600}$ readings for 36 hours. The parameters were orbital shaking for 30 seconds, frequency of 559 cpm (3mm), delay of 100 m/sec, set point of 37°C. The $O.D._{600}$ readings were recorded after 30-minute time intervals of orbital shaking.

4.0 Results and Discussion

4.1 Unsaturated PGs Present in S. aureus JE2 with FA Supplementation

JE2 was supplemented with 10-HSA, *cis*-VA, *cis*-9,10-MOA, OA, and EtOH. The unsaturated PGs are shown in Figure 4.1. There are unsaturated PGs present in each of the samples except the ones supplemented with EtOH. These results show that *fakA::Tn* is phosphorylating the FA, integrating them into the membrane as a phospholipid, and elongating them through the FASII pathway. PG 32:1 occurs from the combination of endogenous FA 14:0 with the exogenous *cis*-VA or OA. The presence of PG 32:1 in *S. aureus* supplemented with 10-HSA is likely from OA as well, since OA can be produced from 10-HSA by the reverse activity of OhyA. There is no PG 32:1 in the bacteria supplemented with *cis*-9,10-MOA because it is a 19-carbon fatty acid. However, the combination of *cis*-9,10-MOA with FA 15:0, the most abundant endogenous FA in *S. aureus,* results in the high abundance of PG 34:1. The combination of the 18-carbon exogenous FAs, 10-HSA, *cis*-VA, and OA, with endogenous FA 15:0 results in PG 33:1, while *cis*-9,10-MOA combines with FA 14:0 to produce PG 33:1. The presence of PG 35:1 is evidence of elongation through FASII, as the 18:1 exogenous FAs are elongated to 20:1 and combined with FA 15:0. These lipidomics data confirm that the various structures of unsaturated fatty acids can be phosphorylated by fakA and incorporated into membrane PGs, with or without elongation by FASII.

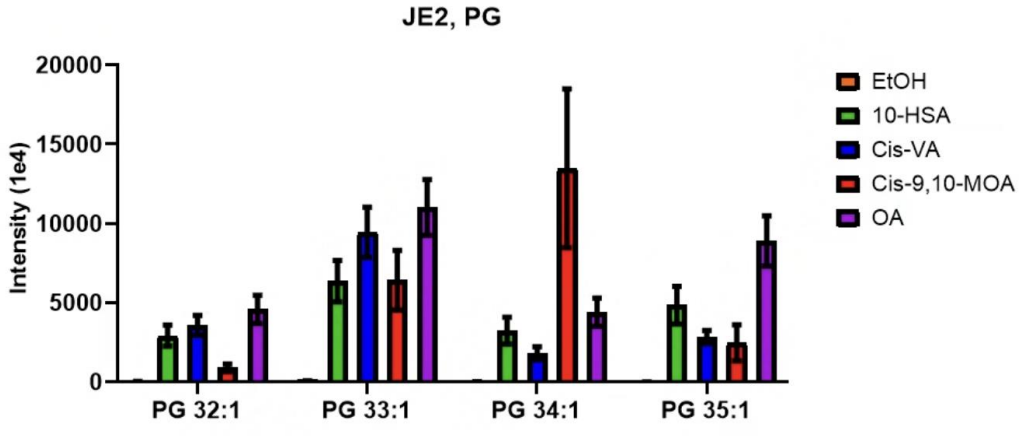

Figure 4.1. Unsaturated PGs Present with the Supplementation of 10-HSA, *cis*-VA, *cis*-MOA, and OA

4.2 OhyA Impacts OA and Not cis-VA or cis-9,10-MOA

We next evaluated the presence and abundance of 10-HSA in all bacteria cultured with exogenous FAs to confirm the specificity of OhyA for cis-geometry double bonds in the Δ9. The abundance of each exogenous FA and 10-HSA is shown in Figure 4.2 for all the FA-supplemented conditions. As expected, 10-HSA was only detected in the conditions where bacteria were provided OA or 10-HSA. These results confirm that OhyA is highly specific for the OA and cannot act on *cis*-VA or *cis*-9,10-MOA.

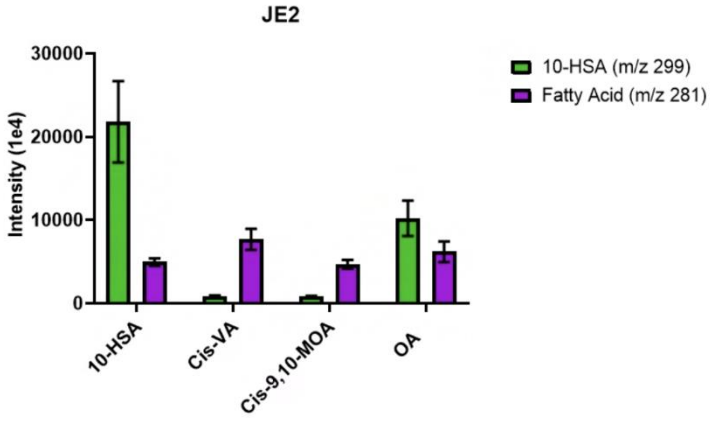

Figure 4.2. Abundance of 10-HSA in each JE2 and FA Sample.

4.3 Influence of OA Analogs on Daptomycin Tolerance of S. aureus JE2 and fakA::Tn

To determine whether the protective effect against daptomycin was unique to OA, we performed daptomycin lag-phase extension assays on *S. aureus* JE2 and *fakA::Tn* in the presence of the OA analogs 10-HSA, *cis*-VA, and *cis*-9,10-MOA. Figure 4.3 shows the impact that *cis*-VA had on the growth of JE2 and *fakA::Tn* when challenged with 15 or 30 µg/mL of daptomycin. While the *fakA::Tn* mutant appears to be inherently more tolerant of daptomycin, 30 µg/mL of daptomycin is sufficient to inhibit the growth of both JE2 and *fakA::Tn* in the absence of an exogenous FA. However, the presence of *cis*-VA enabled the *fakA::Tn* mutant to grow in 15 µg/mL of daptomycin after a short delay in entering the lag-phase. The growth of JE2 was more stunted by daptomycin, even in the presence of *cis*-VA.

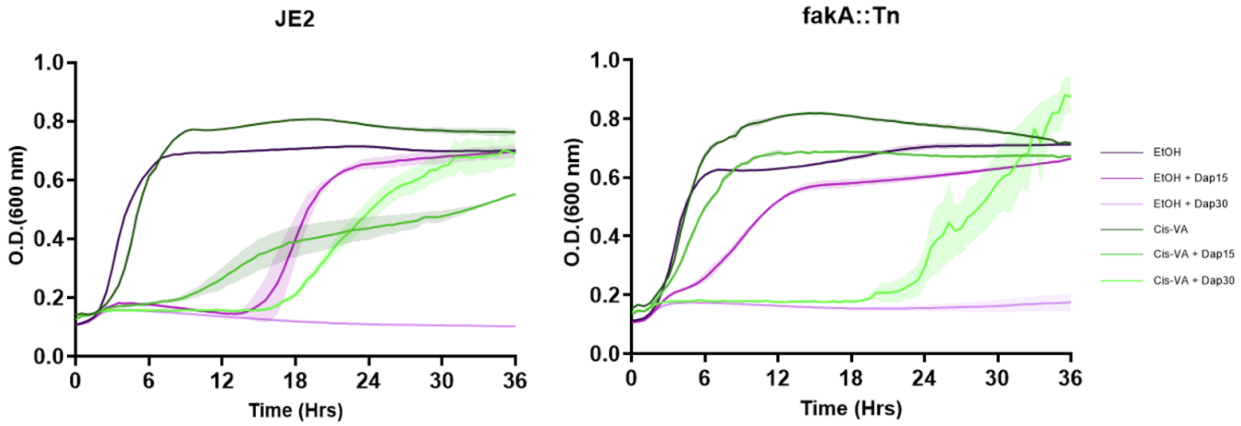

Figure 4.3. *S. aureus* Growth in the Presence of *cis*-VA and Daptomycin.

Figure 4.4 shows the impact that *cis*-9,10-MOA had on the growth of JE2 and *fakA::Tn* when challenged with 15 or 30 µg/mL of daptomycin. Similarly to the previously described *cis*-VA, the *fakA::Tn* mutant appears to be more tolerant to low levels of daptomycin exposure, as seen with the 15ug/mL, yet at higher levels of daptomycin, this effect is reverse not conferring resistance to the bacteria. Within the JE2 sample, the addition of the fakA protein is seen to allow for adaptation and growth of the bacteria both at the 15 and 30 µg/mL concentrations.

While there were some struggles with growth at 15 µg/mL, it is evident that incorporation of *cis*-9,10-MOA is more important for resistance than its presence alone.

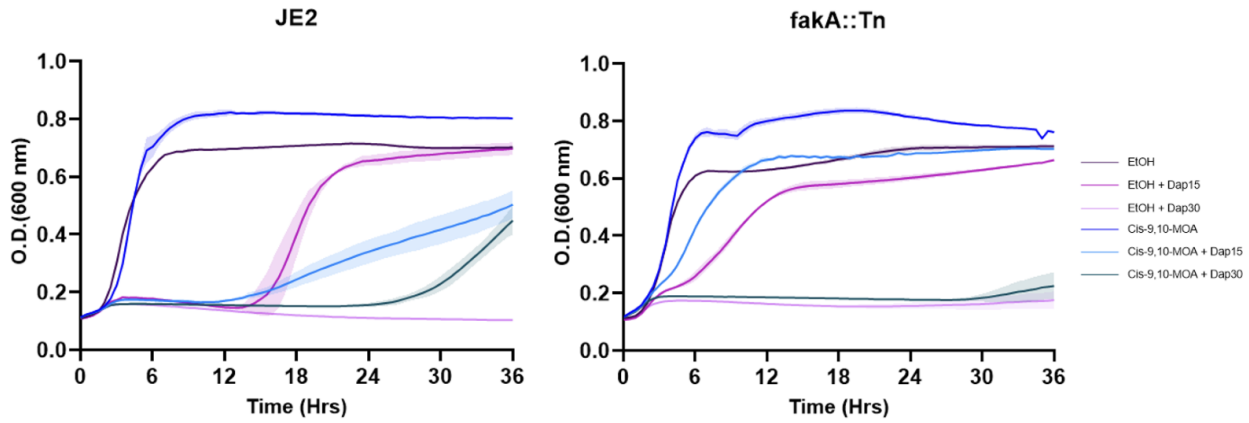

Figure 4.4. *S. aureus* Growth in the Presence of *cis*-9,10-MOA and Daptomycin.

Figure 4.5 shows the impact that 10-HSA had on the growth of JE2 and *fakA::Tn* when challenged with 15 or 30 µg/mL of daptomycin. As additionally seen within Figure 4.3 and 4.4, JE2 and *fakA::Tn* mutant seem equally tolerant of daptomycin. However, 30 µg/mL of daptomycin is sufficient to cause a delay in the lag phase in JE2 and *fakA::Tn*. Compared to c*is*-VA and c*is*-9,10-MOA, the supplementation of 10-HSA with daptomycin resulted in more promotion of resistance to daptomycin. Additionally, compared to the other supplemented species, both the incorporation into phospholipids along with general presence in the bacterial membrane are able to confer resistance to higher concentration of daptomycin. This could be from the presence of the hydroxyl group. We are unaware of how that functional group truly impacts the resistance to daptomycin.

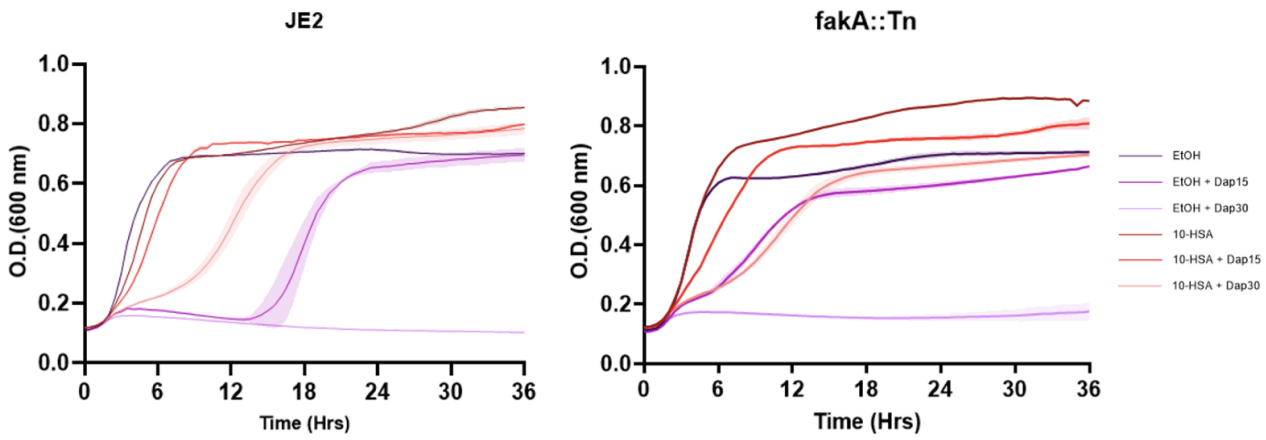

Figure 4.5. *S. aureus* Growth in the Presence of 10-HSA and Daptomycin.

5.0 Conclusions

The presence of OA promotes daptomycin resistance as shown in Figure 1.2. The presence of certain unsaturated PGs is because of the presence of fakA and the supplementation of FA. OhyA hydrates OA to produce 10-HSA, but this reaction doesn't occur with any other FA. The incorporation of *cis*-VA and *cis*-9,10-MOA promotes daptomycin resistance in JE2. The presence of *cis*-VA, Cis-9,10-MOA, or another process within the cell promotes daptomycin resistance in *fakA::Tn*. The presence of 10-HSA strongly promotes daptomycin resistance, potentially because of the structural differences between 10-HSA and the other FA.

6.0 References

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